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Serum Activin-A as a Potential Biomarker for early Stage Ulcerative Colitis (UC): An Immunological and Molecular Evaluation Study using Murine Animal Model

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ABSTRACT

Ulcerative colitis (UC), a form of inflammatory bowel disease (IBD), is a chronic tissue-destructive disorder of the colon. Current laboratory diagnostic assays depend on evaluation of calprotectin that boost with the advancement of the diseases. The present study aimed to evaluate activin-A as a potential alternative marker for early detection of ulcerative colitis (UC). Experimental murine models of short-term versus long-term dextran sodium sulphate (DSS)-induced UC were developed and assessed histopathologically for evaluation purposes. Immunoassays were adopted for evaluation of both activin-A and calprotectin levels in collected serum and fecal specimens using commercial ELISA kit. Colon tissue specimens were homogenized and used for evaluation of activin-A and calprotectin gene expression using quantitative real-time PCR. The results revealed significant increases in serum and fecal activin-A in both short-term and long-term colitis models while there was a significant increase of fecal calprotectin only in long-term UC. Evaluation of gene expression revealed significant upregulation of activin-A in short-term but not in long-term UC model, whereas calprotectin expression showed significant upregulation in long-term but not in short-term of UC model. Interestingly, strong positive correlation was evident between serum, fecal and gene expression of activin-A in both short-term and long-term UC models. On the other hand, calprotectin showed negative correlation between serum and fecal levels in long-term model and positive correlation between serum and gene expression levels in short-term UC. In conclusion, while fecal calprotectin is a good indicator for late stage UC, serum activin-A is a potential biomarker that promote early prognosis of UC.

INTRODUCTION

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD) which share similarities with Crohn's (CD) disease and microscopic colitis **(Jegadeesan *et al.*, 2013).** UC is defined as a chronic tissue-destructive disorder characterized by chronic inflammation and mucosal ulceration confined to colon and usually accompanied by abdominal pain and diarrhea that may contain mucus, blood, or pus **(Awaad *et al.*, 2018).**

It was proposed that UC is caused by an abnormal mucosal immune response that is facilitated or initiated by microbial factors and epithelial cell abnormalities **(Strober *et al.*, 2007).** It is now widely accepted that a complex interplay of genetic predisposition, environmental trigger, and abnormal immune response contributes to disease initiation and progression **(Mayer, 2010).** UC exhibit distinct cellular responses in the context of intestinal inflammation **(Sartor, 2006).**

Many ethical and practical considerations limit human and domesticated animal studies of UC. Difficulties in controlling variables as well as inability to demarcate the early stages of the disease are added limitations. Therefore, animal modelling of the disease is an invaluable alternative tool for studying the pathogenesis of the disease. Animal models play a key role in understanding complicated diseases such as UC **(Catana *et al.*, 2018).**

Animal models of IBD and UC, based on induction method, are generally divided into as either spontaneously induced, genetically-modified, adoptive transfer or chemically induced models **(Kiesler *et al.*, 2015).** Chemically-induced UC in rodents is the most commonly adopted model for studying UC mainly for being fast, economic and effective strategies for induction **(Jiminez *et al.*, 2015)**. The most widely used UC model employs dextran sodium sulfate (DSS) to induce epithelial damage. The DSS-induced colitis model allows for UC investigation due to its rapidity, simplicity, reproducibility and controllability. The ease of controlling the severity as well as the staging range from acute to chronic stage of the disease model via adjustment of DSS dosage and administration duration protocols are additional advantages of the model **(Eichele and Kharbanda, 2017)**. The simplicity to administer DSS in the drinking water is another preference factor for choosing such model **(Ghattamaneni *et al.*, 2019).** It was reported that dose and duration of DSS administration could influence the degree and stage of UC. As administration of 5% DSS for one week was reported to induce an acute form of UC, which is clinically undiscoverable, while longer administration period up to 4–8 weeks resulted in induction of the clinically known chronic form of the disease **(Eichele and Kharbanda, 2017)**.

Accurate and early diagnosis of UC is of great value for the treatment and control of the UC. Laboratory biomarkers are noninvasive indices that allow for disease assessment in order to improve its management. In this respect, calprotectin, a calcium- and zinc-binding protein consisted of two small anionic proteins, is a widely used fecal marker for UC. Fecal calprotectin (FC) has been broadly used as a fecal biomarker of IBD and UC**.** In response to GIT inflammation, neutrophils influx-derived calprotectin is secreted into the intestinal lumen and hence excreted in the stool indicating the gut inflammation **(Kopi *et al.*, 2019)**

Several studies documented the efficiency of fecal calprotectin in evaluating UC activity and its post treatment response as well as in envisaging its relapse **(Mumolo *et al*., 2018).** Recently, serum calprotectin has been proposed as a promising serum biomarker that is more convenient for monitoring UC patients in routine practice. Assessment of serum calprotectin revealed strong correlation with its fecal level **(Leach *et al*., 2007)**. However, the significance of serum calprotectin as a potential biomarker for UC evaluation is controversial as other studies reported that serum calprotectin was correlated significantly with other non-specific inflammatory markers as CRP and albumin but not with fecal calprotectin **(Hare *et al*., 2013; McCann *et al*., 2017).** Therefore, further investigations are required to validate the value of serum calprotectin as well as other serum markers.

Activins are members of the Transforming Growth Factor-β (TGF-β) superfamily. They hence play a regulatory role of wide range of vital physiological processes including embryonic development, cell differentiation, inflammatory conditions and apoptosis in various tissues **(Massagué, 1990)**. Based on type of its beta β -subunits (βA and βB), activins are classified into three different isoforms. This included either homodimer isoforms as activin-A (two βA subunits) and activin-B (two βB subunits) or heterodimer as activin-AB (one βA and one βB subunits) **(Roberts and Sporn, 1991).**

Interestingly, activin-A are recently showed to play a mediator role in acute and chronic inflammatory conditions such as asthma, sepsis, and inflammatory bowel disease via its potent pro-inflammatory actions with subsequent destructive inflammatory responses induced by released pro-inflammatory cytokines **(Hedger *et al*., 2011).** Moreover, several studies have demonstrated the expression of activin-A, and its receptor in differentiated epithelial cells of the villus region of GI tissues. Using in situ hybridization, the highest levels of activin-A mRNA was evident in the mucosa and submucosa of damaged intestinal regions with massive accumulation of inflammatory cells. **(Yardley, 1986; Munz *et al*., 1999; Werner, 1999; Sonoyama *et al*., 2000)**.

Since activin-A is an important early releases pro-inflammatory mediator in several inflammatory conditions including inflammatory bowel dieses, we hypothesized that measurement of serum level of activin-A could play a significant role in early and accurate assessment of ulcerative colitis. To test this hypothesis, the current study evaluated serum level of activin-A in correlation to its fecal and tissue expression levels as compared to that of calprotectin using DSS-induced murine model of short-term versus long-term ulcerative colitis.

MATERIALS AND METHODS

2.1. Animals

A total of 20 apparent healthy Wistar albino female rats (3-4 months old; weight, 200±20 g) had been obtained from the experimental animal house, Department of Pathology, Faculty of Veterinary Medicine, Assiut University, Egypt. Rats were maintained in an environment with a 12/12 - light/dark cycle at 21.0±2.0 ˚ C (room temperature) and 60.0±5.0% relative humidity (RH). The rats were kept for 2 weeks to be acclimatized on the new environment and were fed on a standard rat pellet diet and clean tap water ad libitum, followed by fasting for 12 h prior to the experiment.

2.2. Experimental protocol.

The experiment was performed at the experimental animal house of of the Forensic Medicine Department, Faculty of Veterinary Medicine, Assiut University, Egypt. Dealing with the experimental animals, collection of samples and euthanasia were done according to the regulations for animal care and welfare that postulated with the Ethical committee at the Faculty of Veterinary Medicine, Assiut University, Egypt.
The rats were randomly divided into 4 groups; each group composed of five animals and kept in separate cages. Group I represented short-term UC model that mimic the early stage of UC, Group II represented long-term UC model that mimic the late stage of UC. While, Groups III & IV were kept as negative control groups for the current study.

2.3. Induction of ulcerative colitis models.

Animal model of ulcerative colitis was induced using 3% dextran sodium sulphate (DSS, MW 40,000, Thermo Fisher Scientific Inc.) as previously described **(Arda-Pirincci and Aykol-Celik., 2000, Roy *et al*., 2020),** with some modifications. Briefly, acute model of UC was induced using 3% DSS that was added to drinking water for six successive days. Chronic model of UC was induced with 3% DSS for three cycles. Each cycle consisted of 3 days on tap water contained 3% DDS followed by 12 days on tap water without DDS. The two negative control groups received tap water without treatment for 6 days and 45 days, respectively.

2.4. Collection of samples

2.4.1. Blood samples

At the end of the experiment (six days for acute UC model and 45 days of chronic UC model), rats of all groups were fasted overnight and subsequently were euthanized under complete anesthesia by Isoflurane inhalation anesthesia using drop jar method. Two ml of whole blood were collected from each rat directly from the heart by cardiocentesis after dissecting of rats in a plain tube. The samples were coagulated at room temperature for 10-15 minutes, centrifuged at the speed of 3000 Xg for 20-min. Serum were then collected and stored in -20°C till used.

2.4.2. Fecal samples

Whole colon from rectum to caecum were resected gently, fecal content was emptied into plastic cups. 0.5 g the collected fecal samples were diluted by 2 ml phosphate buffer solution (PBS) (0.1 M, pH 7.2). It is just a qualitative dilution to allow pipetting of fecal suspensions. Fecal debris were discarded by centrifugation 1500 Xg for 2 minutes then the supernatant was stored in -20°C till used.

2.4.3. Tissue samples

The colon was flushed with cold potassium phosphate buffer (0.1 M, pH 7.2) to remove residual bowel contents and slit opened longitudinally. Colon specimens were divided into two parts, one part was processed for histopathological examination, while the other part was kept in RNAlater for further quantitative gene expression. The opened colons for

histopathological examination were then submerged overnight in 10% (v/v) neutralized formalin with the mucosa on the upper side between layers of filter papers.

2.5. Enzyme linked immunosorbant assay (ELISA):

The fecal suspensions and serum samples were thawed in room temperature, reagents and equipment were prepared as instructed for evaluation of Activin-A and Calprotectin levels using commercial ELISA kit for rat Calprotectin and rat Activin-A (SinoGeneClon Biotech Co., Ltd) in different model groups as compared to control ones. Procedure of measurement was executed according to the manufacturer's instructions. Briefly, 50 µl of each test standard (provided) and 10 µl from each tested samples plus 40 µl of provided diluent were added to corresponding plate wells. The plate was covered with adhesive strip then was incubated for 30 min at 37 C in digital thermostatic shaker DTS-2 (system digital thermostatic shaker, DTS-2 Skyline). The plate was then washed by pipetting washing buffer to every well, left for 30 s then drained. This step was repreated 5 times by using ELISA washer (system washer Hydro Control, Tecan, Austria). Then, 50 µl of HRP-conjugate reagent were added to each well, except blank well. The plate was then covered with adhesive strip, incubated for 30 min at 37 C in digital thermostatic shaker DTS-2. The plate was then washed as described earlier. Then, 50 µl of chromogen solution A, and 50 µl of chromogen solution B were added to each well followed by incubation for 15 min at 37 C in digital thermostatic shaker DTS-2. At the end of incubation period, 50 µl of stopping solution was added to each well. Finally, the absorbance was read by ELISA reader microplate spectrophotometer (sunrise absorbance reader, Tecan, Austria) using 450nm wavelength filter.

2.6. Histopathological examination:

Collected tissue specimens were fixed in 10 % neutral buffered formalin. Then dehydration by ascending grades of alcohol, clearing by xylene, embedded in paraffin and sectioned at 4-5 µm, and stained by haematoxylin and eosin (H&E**) (Banchroft *et al*., 1996)**. Prepared tissue sections were examined and evaluated by an expert histopathologist for reporting and confirmation of acute and chronic changes in tissues of induced model groups as compared to corresponding control groups.

2.7. Quantitative real time PCR

Total RNA was isolated from the stored colonic specimens in RNALater following homogenization of the specimens and by using Purelink RNA mini kit from Life Technologies (Thermo Fisher Scientific, CA, USA) according to the manufacturer's instructions. During the extraction protocol, extracts were treated with RNAse-free DNAse to avoid contamination with genomic DNA. Total extracted RNA concentration was measured using Gene Quant 1300 Spectrophotometer (Biochrom a division of Harvard Bioscience, Inc, USA) and its integrity and quality was evaluated through the A260/A280 ratio. cDNA synthesis was conducted using Reverse Transcription Kit from Applied Biosystems, (Thermo Fisher Scientific, Warrington, UK) as described by the manufacturer. Following cDNA synthesis, quantitative RT-PCR of Activin-A, Calprotectin and GAPDH

genes was conducted with primer sets listed in Table 1 using Mx 3000P stratagene Real Time PCR system (Agilent, USA) in duplicate wells. Reaction were conducted using power SYBR green master mix from Applied Biosystems, (Thermo Fisher Scientific, Warrington, UK). Reaction condition included total reaction volume and thermal profile for each gene. Total reaction volume for all 3 genes was 15 µl that included 7.5 µl SYBR green master mix, 1µl of each primer and 5.5 µl cDNA sample. Thermal profiles of Real-time PCR that were used for the detection of Activin-A (Inh.A), Calprotectin and GAPDH were shown in **table 1**. The results were normalized against the Ct values of GAPDH house-keeping gene. Relative gene expression was measured by the 2-ΔΔCt method and expressed as fold-change compared with the normal control group as previously described **(Refaat *et al.*, 2015).**

3. Statistical analysis

Data were expressed as Mean ± SD, Statistical analysis was conducted using SPSS 13.0 for windows (SPSS, Chicago, USA). The difference in serum, fecal levels and gene expression of Activin-A and Calprotectin were compared using one-way ANOVA followed by least significant difference (LSD) post-hoc analysis ($p<0.05$). Pearson correlation between percent of increase of fecal, serum and gene expression of Activin- A and Calprotectin in short and long-term treatment was conducted using SPSS 13.0 for windows (SPSS, Chicago, USA). Percent of change of Activin-A and Calprotectin gene expression as well as their fecal and serum levels were calculated as previously described (Arda-Pirincci, P. & Aykol-Celik, G. 2000.) using the following equation.

$$percent\ of\ change = \frac{value\ of\ individual\ in\ group\ of\ interest\ -\ value\ of\ control\ individual\ for\ this\ group}{value\ of\ control\ individual\ for\ this\ group}\ x\ 100$$

4. Ethical Approval

Bioethical approval of the current study was sought from the Bioethical committee of Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, **(no. 06/2032/0056)** according to The OIE standards for use of animals in research and was hereby authorized to conduct scientific tests, experiments or investigations involving the use of living vertebrate animals.

RESULTS

3.1. Gross findings

Gross examination of the examined GITrevealed no apparent inflammatory signs in the acute group as compared to the control group. Nevertheless, some inflammatory signs were apparent on colons of the long-term model group animals as compared to those of the control group (Fig. 1). Physical examination of fecal matter revealed no apparent changes in the short-term model of induced UC. However, the fecal matter of long-term model showed changes of consistency with increased moistening and one rat only showed bloody feces (Fig. 2).

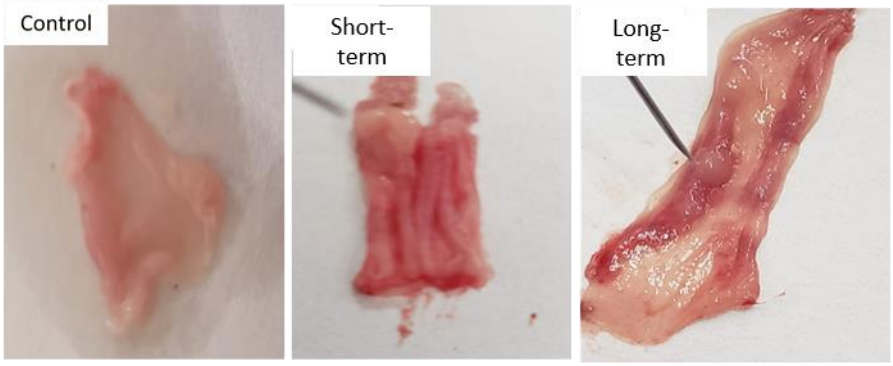

Figure 1: The obtained photos showed gross examination of the colon in different rat groups Control and short term models showed no apparent inflammatory changes while the long-term model showed congestion, erosion and other inflammation signs.

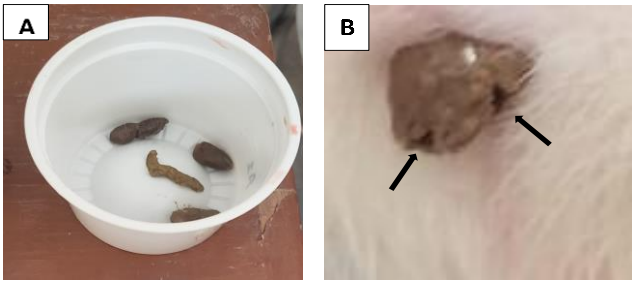

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Figure 2: The obtained photos showed gross examination of fecal matter. Picture (A) showed normal feces of the control rat, picture (B) showed bloody feces in the long-term model of the UC rat.

3.2. Histopathological characterization of the induced UC models:

Microscopic examination of the colon of the control group showed normal colon mucosa which lined by simple columnar epithelium with goblet cells, submucosa, muscular layers and serosa were also normal. The group of rats with induced short-term model of UC showed ulceration of the colon mucosa with disruption of the lining epithelium. Vascular changes were more pronounced in the form of congestion of the submucosal blood vessels and edema. Inflammatory cell infiltration was evident in the form of macrophages and lymphocytes (Fig. 3). Examination of rat colon in long-term model group revealed extensive ulceration of the colon mucosa with disruption of the epithelium and hyperplasia of goblet cells. Heavy lymphocytic infiltration was remarkable in lamina propria, submucosa and muscular layer (Fig. 4).

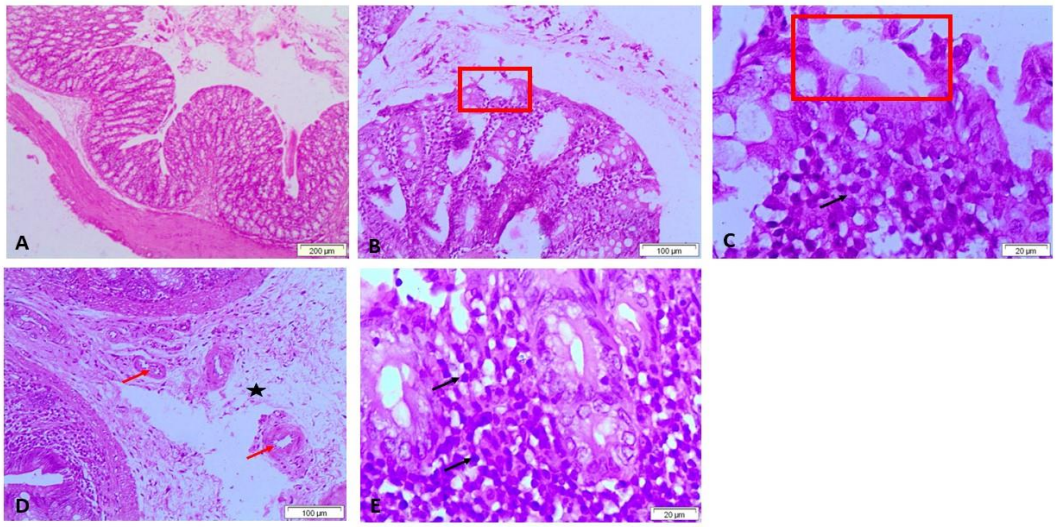

Figure 3: Representative micrograph of the rat colon (X 100 magnification), A) Control negative group showing normal colon mucosa, submucosa, muscular layers and serosa. B-E) Short-term UC model group showing ulceration of the colon mucosa with disruption of the epithelium (Red rectangle), Congestion of the submucosal blood vessels (Red arrows), edema in the submucosa (Star) and inflammatory cell infiltration (Black arrows). H&E stain.

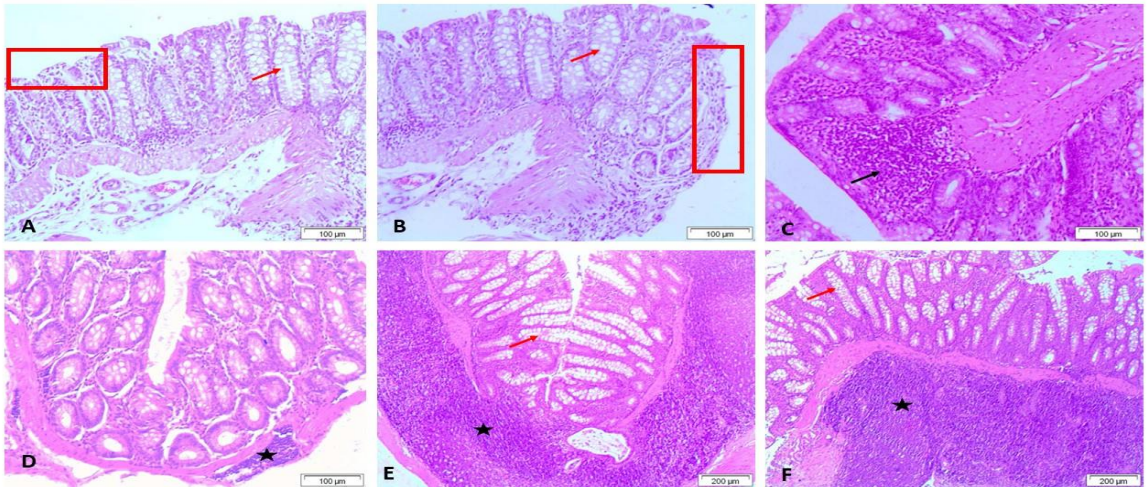

Figure 4: Representative micrograph of the rat colon (X 200 magnification) in long-term UC model group showing prominent ulceration of the colon mucosa with disruption of the epithelium (Red rectangle), hyperplasia of goblet cells (Red arrows), lymphocytic infiltration in lamina propria (Black arrow) and heavy lymphocytic infiltration in submucosa and muscular layer (Stars). H&E stain.

3.4. Enzyme linked immunosorbent assay (ELISA) results

3.4.1. Fecal levels of activin-A and calprotectin

There was a significant increase in fecal activin-A level in both short (P=0.04) and long-term model (*P* < 0.0001) groups compared to the control group (74.32±17.85 pg/ml). Notably, fecal activin-A level in the long-term group (210.74±37.62 pg/ml) showed a significant increase (*P*=0.02) compared to the short-term group (124.78±44.432 pg/ml). On the other hand, fecal calprotectin showed significant increases in long-term model (167.30±22.745 ng/ml) compared to the acute (117.00±27.31 ng/ml) (P=0.006) and control (89.82±22.16 ng/ml) (*P* < 0.0001) groups, while there was no significant difference between short term model and control (*P*=0.101) groups (Figure 5).

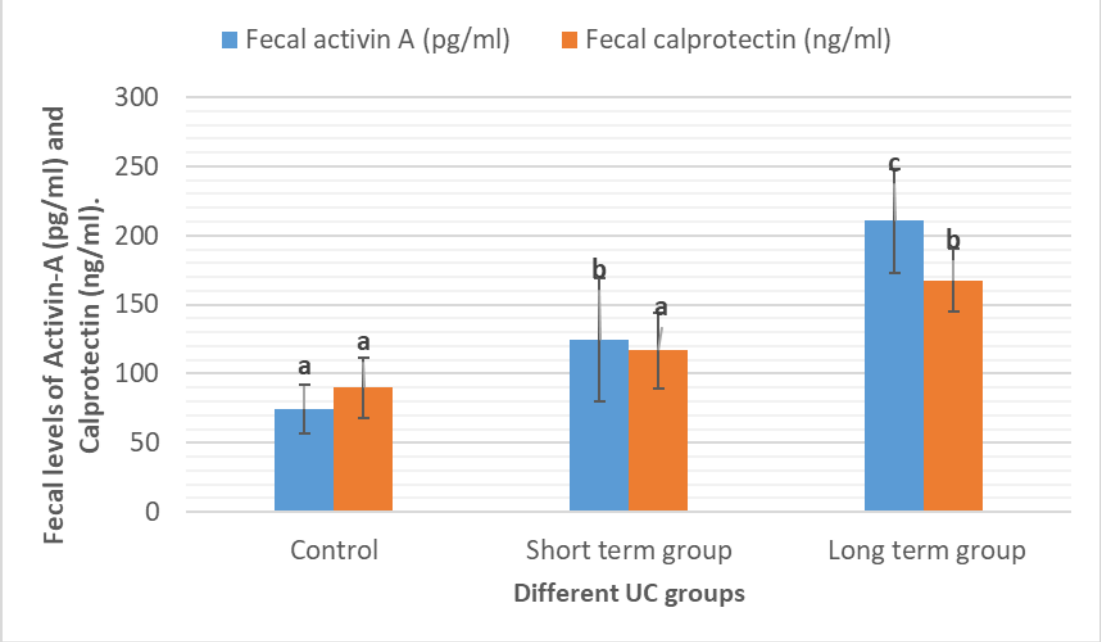

Figure 5 : Fecal levels of activin-A and calprotectin in different UC groups (Different letter symbols indicate significance, while similar letters indicate no significance)

3.4.2. Serum levels of activin-A and calprotectin

The mean serum activin-A in the control, short term and long-term groups were 37.18±5.01, 50.56±6.23 and 60.32±12.80 (pg/mL), respectively. There was a significant increase in serum activin-A level in both long-term group (*P*=0.039) and short-term group (*P* =0.002) compared to control group while there was no significant difference between serum activin-A level in the short-term and long-term group (*P*= 0.102**),** (Figure 6)**.**

The mean level of serum calprotectin in the control, short-term and long-term groups were 36.92±4.33, 38.35±4.57and 41.65±4.12 ng/mL, respectively. Serum calprotectin level showed no significant differences between different groups (*P*=0.250), (Figure 6).

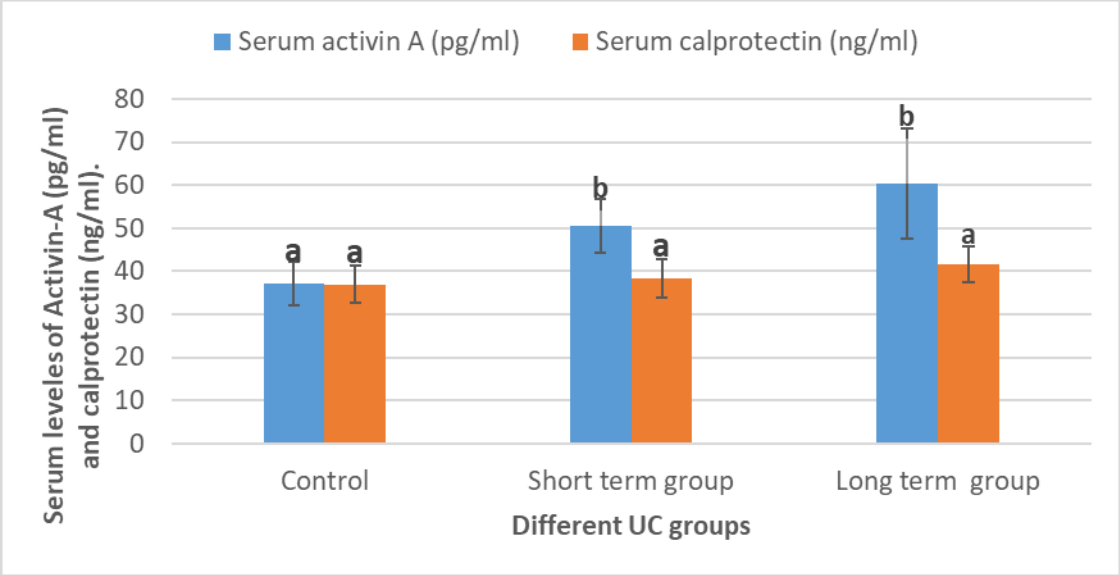

Figure 6: Serum levels of activin-A and calprotectin in different UC groups. (Different letter symbols indicate significance, while similar letters indicate no significance)

3.5. Evaluation of selected gene expression by quantitative RT-PCR:

Evaluation of gene expression levels of *INHBA* and calprotectin were conducted based on their relative mRNA expression patterns as compared to that of GAPDH as housekeeping gene by qRT-PCR. Current findings revealed a significant up-regulation of *INHBA* gene expression in the induced short-term model of the UC group (Mean± SD 24.54 ± 16.68) (*P* =0.003) and (*P*=0.006) as compared to both control group (Mean± SD 1.30 ± 1.03) and long-term model group (Mean±SD 4.06 ± 2.09), respectively. The *INHBA* expression in the long-term model showed more than 3-fold increase as compared to the control group, yet statistically remained non-significant (*P*=0.662), (Figure 7).

On the other hand, relative expression of calprotectin showed non-significant (*P* =0.893) upregulation in the short-term model group (Mean± SD, 1.41 ± 0.94) as compared to the control group (Mean± SD, 1.23 ± 0.70). However, relative calprotectin expression showed significant upregulation (*P* =0.010) and (*P*=0.012) in the long-term model group (Mean ± SD, 5.31 ± 3.44) as compared to the control group (Mean± SD, 1.23 ± 0.70) and the short-term group (Mean± SD, 1.41 ± 0.94), respectively (Figure 7).

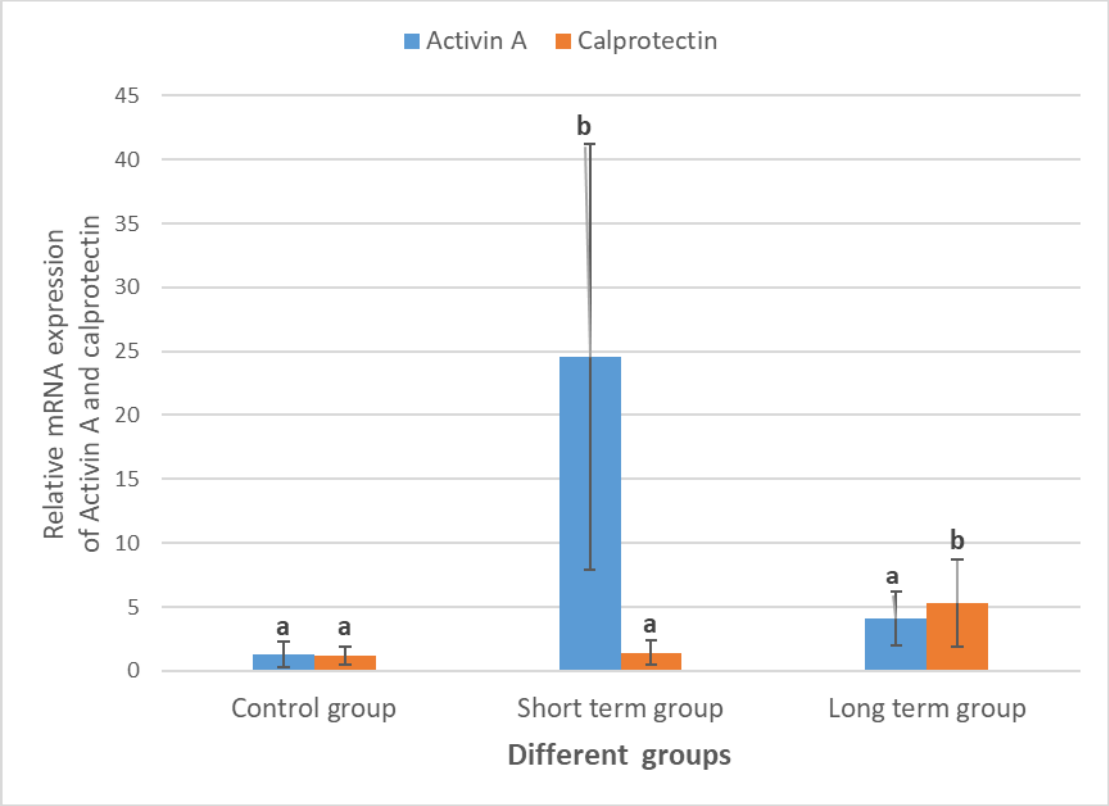

Figure 7: Activin-A and calprotectin gene expression in different UC groups. (Different letter symbols indicate significance, while similar letters indicate no significance)

3.6. Correlation findings between gene expression, fecal level and serum level of activin-A and calprotectin.

3.6.1. The short-term UC model:

There was a strong positive correlation between gene expression of activin-A and serum activin-A (r=0.981, P=0.028). Furthermore, the same strong correlation was noted between activin-A gene expression and fecal activin-A (r= 0.901, P =0.037). In addition, fecal activin-A was strongly correlated with serum activin-A (r=0.988, P=0.002) (Table 1).

Regarding percentage of increase of calprotectin in the short term model of UC, calprotectin gene expression was positively correlated with serum calprotectin and negatively with fecal calprotectin (r=0.951, P=0.013) (r= -0.896, P =0.039), respectively. In addition, there was a negative correlation between fecal calprotectin and serum calprotectin (r= -0.983, P=0.003), (Table 2).

3.6.2. The long-term UC model:

There was a strong positive correlation between percentage of increase of activin-A gene expression and fecal Ativin-A (r= 0.901, P= 0.037). Furthermore, the same strong correlation was noted between activin-A gene expression and serum activin-A (r=0.918, P=0.028). In addition, fecal activin-A was strongly correlated with serum activin-A (r=0.998, $P < 0.0001$), (Table 3). While, there was no correlation between calprotectin percentage of increase in gene expression, fecal and serum (Table 4).

DISCUSSION

Ulcerative colitis (UC) is a chronic inflammation of the colon that associated with ulcers of the colorectal mucosa, accompanied by abdominal pain and diarrhea which may be with mucus, blood, or pus secretion **(Awaad *et al*., 2018)**. UC is an important public health problem, which could lead to peritonitis and increase the risk of progress to colorectal cancer if left untreated **(Johnson *et al*., 2020)**. Current laboratory evaluation of UC progression rely on calprotectin that currently used as a proved fecal biomarker for assessment of UC **(Kopi *et al*., 2019)**. Fecal calprotectin is efficient in IBD monitoring, disease activity, and response to therapy as well as in predicting relapse **(Mumolo *et al*., 2018).** Activin-A is an early released tissue and blood biomarker for many inflammatory condition such as sepsis and inflammatory bowel disease **(Hedger *et al*., 2011).**

The present study, evaluated The validity of using activin-A as a serum biomarker for early diagnosis of ulcerative colitis in correlation with its fecal level as well as its gene expression level in colon tissue as compared to the currently used biomarker (calprotectin) using murine model of DSS induced short-term and long-term models of ulcerative colitis.

With regard to experimentally developed murine models of UC, obtained gross and histopathological changes were well-matched with long-term versus short-term inflammatory conditions as previously described **(Awaad *et al*., 2018; Catana *et al*., 2018; Ghattamaneni *et al*., 2019)** and confirm the validity of induced models of the study.

The significant increase of activin-A at both fecal and serum levels as revealed in the current study is consistent with the findings of previous studies that documented the elevation of serum and tissue concentrations of activin-A during early stage of infection and inflammation. Significant elevation of plasma activin-A levels was reported as early as 3 days post-induction by 5% DSS in mice model of colitis **(Zhang *et al*., 2009)**. Similar findings were documented in other inflammatory conditions where significant increase of activin-A were evident during acute lung injury **(Jones *et al*., 2007)**. It was also reported that elevated circulating levels of activin-A was found predictive to progression of human and experimental models of septicemia **(Woodruff, 1998)** . Moreover, the very early release of activin-A during inflammatory condition was documented by the rapid elevation of its serum level till reached the peak in 40-50 min post-injection with bacterial lipopolysaccharide (LPS) **(de Kretser *et al*., 2012)**. Other studies also reported the increases activin-A production in monocytes and mouse peritoneal macrophages post-LPS injection **(Ogawa *et al*., 2000)**. These findings provided the evidence of activin-A role in

response to acute inflammatory condition**.** The fact that luminal microflora plays a crucial role in the development of colitis, suggests that early released activin-A as documented in current study is possibly a result of direct stimuli from the pathogen-derived inflammatory signal, or an indirect effect from other cytokines; further investigations are required to clarify this assumption.

With Regard to calprotectin, the significant elevation of its fecal level only during the long-term induction as reported in the current study was in general agreement with previous studies that documented the significant elevation of fecal calprotectin in active (late stage) UC as compared to inactive (early stage) UC **(Kopi *et al*., 2019)**. On the other hand, the non-significant changes of serum calprotectin level during both early and late stages of induced UC as documented in the current study was kind of controversial. Some studies reported correlated serum and fecal calprotectin activity during active and inactive UC and therefore suggested calprotectin as a reliable serum marker for monitoring UC and inflammatory bowel disease (IBD) activity **(Mori *et al*., 2021)**. In addition, other studies reported the elevation of serum calprotectin concentration in dogs with idiopathic IBD as well as in humans with ulcerative colitis and Crohn's disease **(Heilmann *et al*., 2012).** On the other hand, other studies, in agreement with current study findings, documented the absence of significant correlation between serum and fecal calprotectin levels in UC patients. On the other hand, a mild significant correlation was also reported between serum calprotectin and C-reactive protein (CRP) levels in those patients. Therefore, it was concluded that serum calprotectin is probably excreted mainly from circulating leukocytes and not from inflamed intestine. Then elevated serum calprotectin was proposed as being a possible result of systemic inflammation, rather than being direct response to inflammatory condition of GIT **(Fukunaga *et al*., 2018)**. Other study also reported the lack of significant correlation between serum and fecal calprotectin and concluded that serum calprotectin is unlikely to be of clinical significance as a blood-based biomarker for monitoring IBD **(McCann *et al*., 2017)**.

Notably, activin-A gene expression revealed highly significant up-regulation (more than 20-fold increase) in induced short-term UC model (Mean± SD 24.54 ± 16.68) (P =0.003) and (P=0.006) as compared to the control group (Mean± SD 1.30 ± 1.03) and the long-term model group (Mean±SD 4.06 ± 2.09), respectively. These findings were in general agreement with previous studies that documented the extensive up-regulation of activin-A levels in colonic tissues of both DSS-induced colitis murine models **(Zhang *et al*., 2009)** and human cases of IBD **(Dignass *et al*., 2002)** as compared to normal colonic tissues. Interestingly, the current study revealed significant down regulation of activin-A gene expression in colonic tissue of the long-term model as compared to that of the short-term model (Figure 7). Comparable findings were reported by previous studies that documented the upregulation of activin- A gene expression during the first 7 days post-injury, with its expression levels dropped near its baseline levels after 13 days **(Hübner *et al*., 1997).** However, the significantly elevated serum and fecal level of activin-A despite its low gene expression level from colonic tissue during the long-term study as revealed in the current study suggest that induced activin-A is possibly due to direct stimuli from inducer-derived inflammatory signal during early stage and due to indirect effect from other inflammatory cells-derived cytokines during late stage of UC. Supported by the

observation of co-expression of activin-A and its receptors in inflamed tissues, activin-A was suggested to exert its effect on both inflammatory cells and intestinal epithelial cells via acting as both autocrine and paracrine factors during colitis **(Zhang *et al*., 2009).** Moreover, the same study also recorded that despite the lack of any significant changes of activin-A expression in colonic epithelial cells by immunohistochemistry, a subset of activin-positive cells were co-localized with macrophage-specific markers, indicating that macrophages represent a significant cellular source of activin during colitis **(Zhang *et al*., 2009).** Several other studies suggested that other factors that are able to activate inflammatory signaling pathways can stimulate activin-A secretion as well. It was reported that activin-A expression is principally induced by activation of the MyD88-dependent signaling pathways linked to the TLRs and to the IL-1 receptor. In response to the activation of these receptors, diverse cell types, most notably cells of the myeloid lineage (monocytes, macrophages and dendritic cells) have been shown to respond by increased production of activin A mRNA and secretion of the activin-A protein **(Akira and Takeda, 2004).** Moreover, upregulation of activin-A expression was reported to be triggered by several pro-fibrotic agents as well such as TGF-β1, tumor necrosis factor-α (TNF-α), endothelin, interleukin (IL)-13, thrombin, and angiotensin **(de Kretser *et al*., 2012).** In addition, non-myeloid cell types have been also shown to stimulate activin-A secretion including hepatocytes, hepato-stellate cells, airway epithelial cells, keratinocytes, endothelial cells, bone marrow stromal cells, neutrophils and T helper type 2 (Th2) cells **(Hardy *et al*., 2010; Hedger *et al*., 2011).**
In conclusion, the current study showed that serum activin-A is significantly increased in both short-term and long-term models of UC. A strong significant positive correlation between serum activin-A and its colon gene expression level as well as its fecal level was evident during short-term model of UC. Interestingly, while serum level of activin-A was significantly increased in long-term model, yet the increase of its colon gene expression level was not significant. This finding would suggest the presence of other source of expression of activin-A during long-term other than the affected colon tissue. Further investigations are required to elucidate this point. On the other hand, only fecal level of calprotectin, not serum level, showed significant increase only during long-term model but not in short-term model. Finally, the current study suggests that while fecal calprotectin is a good indicator for late stage UC, serum activin-A is a potential biomarker that would promote early prognosis of UC.

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Table 1: Thermal profiles of Real-time PCR for detection of Activin-A (*Inh.A*), Calprotectin and GAPDH

		Activin-A (Inh-A)	Calprotectin	GAPDH
Initial Denaturation	**Temp.**	95^{C}		
	Time	5 Min		
Denaturation	**Temp.**	95^{C}	95^{C}	95^{C}
	Time	1 min	1 min	30 S
Annealing	**Temp.**	67^{C}	66^{C}	55^{C}
	Time	50 S	50 S	30 S
Extension	**Temp.**	72^{C}	72^{C}	72^{C}
	Time	50 S	50 S	1Min
Cycles		40		

Table 2: List of primer sets used for qRT-PCR evaluation of Activin-A (Inh.A), Calprotectin and GAPDH including the corresponding genes accession numbers

Gene name	Primer sequence	Gene accession #
Activin-A (*INHBA*)	F- CTCTGTTGACCCACCCCATC R- GATGTTGAGGGCAGCTCGAT	NM_012590.2
Calprotectin	F- AGCTGGAGCGCAGCATAAG R- TGATTGTCCTGGTTTGTGTCCA	NM_053587.2
GAPDH	F- CAATCCTGGGCGGTACAACT R- TACGGCCAAATCCGTTCACA	XM_039107008.1

Table 3: Correlations between percentage of increase of activin-A gene expression and its serum and fecal levels in the long-term UC model

		Activin-A gene expression	Fecal Activin-A	Serum Activin-A
Activin-A gene expression	R	1	.901*	.918*
	P		.037	.028
Fecal Activin-A	R		1	.998**
	P			.000
Serum Activin-A	R			1
	P			

*: Correlation (R) is significant at $P< 0.05$. **: Correlation (R) is significant at $P< 0.01$.

Table 4: Correlations between percentage of increase of calprotectin gene expression and its serum and fecal levels in the long-term UC model

		Calprotectin gene expression	Fecal Calprotectin	Serum Calprotectin
Calprotectin gene expression	R	1	.334	-.278-
	P		.583	.651
Fecal Calprotectin	R		1	-.816-
	P			.092
Serum Calprotectin	R			1
	P			